Analysis of Prostate Tissue DNA for the Presence of Human Papillomavirus by Polymerase Chain Reaction, Cloning, and Automated Sequencing

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We have analysed the DNA from 24 prostate tissue biopsies, spanning a range of Gleason grading from benign to grade 5 and mixed randomly with cervical cancer samples of known human papillomavirus (HPV) status, for the prevalence of HPV DNA, in a double-blind study to ensure complete objectivity. Polymerase chain reactions (PCR) were performed using general E1 open reading frame primers for HPV under low stringency conditions, in addition to reactions containing primers specific for HPV16, E2, and E6 open reading frames under higher, more stringent PCR conditions. The presence of cellular DNA was verified by the use of primers for hypoxanthine guanine phosphoribosyl transferase.

DNA bands were not detected in the prostate biopsies using the HPV16-specific primers under high-stringency PCR conditions, however a predominant band in the 400 bp region was observed in 15 of the prostate biopsies using the general primers and the low annealing temperature of 40°C. This fragment was excised and cloned into the pT7 blue vector and the sequence of the insert determined. Although the cloned sequences initiated and terminated with the two authentic PCR primers, they did not contain a significant HPV-related open reading frame. Our results indicate that HPV type 16 and closely related types, as detected by the general primer pair, are unlikely initiators of prostate carcinogenesis within our population. ***J. Med. Virol. 52:8–13, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: prostate cancer; HPV; PCR; automated sequencing

INTRODUCTION

A clear etiological relationship has emerged between human papillomavirus (HPV) infection and a number of human cancers, most notably carcinoma of the cervix [zur Hausen and De Villiers, 1994]. There has recently been an increasing amount of interest in the male genitalia as both a reservoir for HPV infection and also as a potentially similar target for papillomavirus-mediated tumourigenesis. This has been investigated using highly sensitive DNA detection methods to find HPV DNA in samples of human penile and eurethral cancers [Wiener et al., 1992a,b; Villa and Lopez, 1986], but also in normal asymptomatic urethral cells [Della Torre et al., 1992]. Additionally, a number of papers have reported the detection of HPV genomes in human bladder carcinomas [Anwar et al., 1992a] and human prostate cancers [Ibrahim et al., 1992; Anwar et al., 1992b; McNicol and Dodd, 1991; Rotola et al., 1992b]. In Rotola et al. [1992], an episomal HPV 16 molecule was cloned, and Dodd et al. [1993] were able to detect HPV gene expression in biopsies of human prostate. However, more recent studies [reviewed by Cuzick, 1995] suggest that the detection of HPV in carcinoma of the prostate is less frequent than originally observed and indeed Sinclair et al. [1993] reported that the detection could well be technique specific, in that a variation in the annealing temperatures for the polymerase chain reaction (PCR) used to detect the virus genomes could lead to spuriously positive results.

We wished to test these various options using a double-blind study to ensure complete objectivity and to overcome potential problems of sample cross-contamination in this type of work. Accordingly, human prostatic biopsies taken from both benign and malignant tissue were screened for the presence of HPV DNA. We used individual PCR primer pairs for HPV16 (the most likely high-risk type virus found in genital samples in the United Kingdom) and focussed on the E6 oncogene and the E2 transcriptional control gene. Experiments were also carried out using HPV general primers within

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TABLE I. PCR Results With Prostate Tissue DNA

Clinical sample	Biopsy diagnosis[a]	Gleason grade	Clinical stage	Primer pairs used in the analysis of the DNA			
				HPRT	HPV16-E2	HPV16-E6	General-(E1 ORF)
92/23	B		BPH[b]	+	−	−	+
92/26	B		BPH	+	−	−	+
92/31	B		BPH	+	−	−	+
92/33	B		BPH	+	−	−	−
93/53	B		BPH	+	−	−	+
93/60	B		BPH	+	−	−	−
93/61	B		BPH	+	−	−	−
93/165	B		BPH	−	−	−	−
93/171	B		BPH	+	−	−	−
93/174	B		BPH	+	−	−	+
92/15	M	G1, G3	T3	+	−	−	+
92/27	M	G1	Not done	+	−	−	+
92/41	M	G2, G3	T2	+	−	−	+
92/53	M	G2	T3	+	−	−	+
92/61	M	G2	−	+	−	−	+
92/77	M	G1	T1	+	−	−	+
92/83	M	G3	T3	+	−	−	+
92/84	M	G3	T2	+	−	−	+
92/85	M	G2	T1	+	−	−	Not done
92/86	M	G2	T3	+	−	−	+
93/110	M	G3	Not done	−	−	−	−
93/130	M	G1	T2	−	−	−	−
93/167	M	G3	Not done	−	−	−	−
93/230	M	G2	T1	+	−	−	+
Controls							
Cervical Ca 39				+	+	+	Not done
Cervical Ca 23				+	+	+	Not done
Caski DNA				+	+	+	+
Pure water				−	−	−	−
HPV6 plasmid				Not done	Not done	Not done	+
HPV16 plasmid				Not done	Not done	Not done	+
HPV18 plasmid				Not done	Not done	Not done	+

[a]Samples were sorted into benign (B) or malignant (M) classes according to preliminary histological reports.
[b]BPH, benign prostatic hyperplasia.

the conserved E1 open reading frame of the HPV. The presence of cellular DNA was controlled using the cellular hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene exploited in previous studies in this laboratory [Jalal et al., 1992].

MATERIALS AND METHODS

Patients Studied

Biopsies were obtained by transurethral resection of the prostate TURP (M.S.) from 24 patients at York District Hospital. The biopsies were frozen immediately and stored at −70°C. The data set consisted of 10 benign and 14 malignant samples of varying Gleason grade and TMN staging as determined by the initial histopathological reports (see Table I).

DNA Preparation

DNA was prepared from the biopsies taken in 1992 as described by Maitland et al. [1987], the biopsies being chopped into 2 mm^3 pieces, minced into HIRT buffer (0.01 M Tris-HCl, 0.01 M ethylenediamine tetraacetic acid, pH 8.0, 0.6% sodium dodecyl sulphate) [Hirt, 1967], digested with 200 μg/ml proteinase K overnight at 50°C, and then extracted twice with an equal volume of 1:1 phenol to chloroform mixture. High molecular weight DNA was finally spooled out of the aqueous phase after addition of 1/10 volume of 3 M sodium acetate (pH 4.5) and an equal volume of isopropanol at room temperature to minimise RNA precipitation.

DNA was prepared from biopsies taken in 1993 by embedding the frozen tissues in OCT and cutting 7 μm thick sections with a Leica Cryocut 1800 microtome. The sections were scraped into HIRT buffer and processed as described above.

PCR Amplification

The PCR mixtures using the primers specific for HPV each contained 200 ng of purified sample DNA, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μM of each dNTP, 1.5 mM MgCl, 0.05% (v/v) W-1 detergent, 1.25 units of Taq DNA polymerase (Thermus Aquaticus YT1, Life Technologies, cat. no. 18038-026), 0.4 μM of the relevant specific primer pair, and pure water to a final volume of 25 μl. Conditions for HPV16-specific primer pairs were optimised and designed to detect the E6 [Maitland et al., 1989] and E2 open reading frames (CM Sanders, unpublished). The reaction mixture was overlaid with paraffin oil to prevent evaporation and incubated at 94°C for 5 min to ensure DNA denaturation. Thirty cycles of amplification were then performed, each cycle comprising a denaturation step at 94°C for 1 min, an

annealing step at 50°C for 1 min, and an extension step at 72°C for 1 min. Identical conditions were used for the HGPRT primer pair.

The nonspecific general primer pair GP1 and GP2 [van den Brule et al., 1990], which are located in the strongly conserved E1 open reading frame of the HPV genome, were used as a powerful, nonspecific means of detecting the prevalence of HPV in the prostate DNA. The GP-PCR mixture contained a higher level of $MgCl_2$(3.5 mM), otherwise it was identical to the reaction mixture used for the specific primers mentioned above. The reaction mixture was incubated at 95°C for 5 min prior to 40 cycles of amplification, each cycle comprising a denaturation step at 94°C for 1 min, an annealing step at 40°C for 2 min, and an extension step at 72°C for 90 sec. In addition, an extension step at 72°C for 4 min was introduced after completion of the 40 cycles. The samples were amplified on a Thermal Cycler 480 (Perkin Elmer Cetus), supplied by Applied Biosystems Ltd., U.K. All the PCR products were stored at −20°C after amplification.

PCR Controls

Cervical DNA samples, which had previously been shown to contain low levels of HPV16 DNA [Simons et al., 1995], were introduced and mixed randomly into the prostate sample set as positive controls. The researcher carrying out the PCR was not aware which of the sample sets were positive controls. In addition, DNA from a cervical cell line, Caski, was used as a positive control for HPV16 with the specific primer pair, and three plasmid DNA samples, containing HPV6, HPV16, and HPV18 complete genomes and kindly supplied by H. zur Hausen and E.-M. de Villiers (DKFZ, Heidelberg, Germany), were used as positive controls with the GP1 and GP2 primer pair. The PCR mixture without any DNA present was used as a negative control. All the prostate DNA samples were probed for the presence of cellular DNA prior to the HPV studies taking place, by carrying out a separate PCR amplification using the HRPT primer pair [Maitland et al., 1989].

The biopsy DNA amplification reactions were repeated with the specific HPV16 primer pair four times and with the general (GP) primers three times with varying cycle numbers. The amplifications for HPRT were carried out twice.

Detection of Amplified Products

Ten microlitres of each of the amplified products were analysed by electrophoresis on both 1.5% agarose gels (BRL Ultrapure) and Visigel separation matrix (Stratagene) and the DNA was visualised by ultraviolet fluorescence (UV) after staining with ethidium bromide. Photographs were obtained by means of a Polaroid UV camera system (model CC80, AMS Biotechnology, U.K., Ltd.) and digital images were also obtained on a COHU video camera and system (Vista Vision Systems).

Ligation, Transformation, and pT7Blue Cloning

PCR products from the general primer amplifications were separated in a 1.5% low melting point agarose gel (NuSieve GTG agarose; FMC Bioproducts) and the 400 bp DNA bands were excised with a sterile scalpel blade and then combined in a sterile 1.5 ml microcentrifuge tube. The DNA samples were then purified using the Geneclean II kit (BIO 101 Inc.). Products were then cloned utilising the pT7Blue T-Vector kit (Novagen). Individual white colonies containing the desired recombinant plasmids were selected and plasmid DNA prepared [Maniatis et al., 1989].

DNA Restriction Digestion and Sequencing

The recombinant plasmid DNA was purified using a QIAquick-spin column according to the manufacturer's instructions, quantified by optical density, and analysed by restriction endonuclease digestion using the restriction enzymes Hind III and Bam HI. Plasmid DNA containing an insert was then sequenced on an Applied Biosystems Model 373A Sequencing System using the recommended Taq Dye-Deoxy Terminator cycle sequencing kit and reverse and forward sequencing primers for the pT7blue plasmid (ABI).

RESULTS

DNA from the patients' biopsies was analysed by a series of PCRs using four groups of specific primers. First, a pair of primers specific for HPV16, capable of detecting the E6 gene which is commonly retained in cervical cancers [Baker et al., 1987], was employed. To ensure objectivity, a series of cervical carcinomas previously shown to contain relatively low amounts (about one to five copies per diploid cell) of HPV16 DNA [Simons et al., 1995] were included randomly in a double-blind study such that the operator had no knowledge of the origin of the DNAs to be tested; this was designed to control for both cross-contaminations and specificity. As shown in Figure 1A for a representative number of the samples studied, only the cervical carcinomas produced a positive reaction with the E6-specific primers (lanes 4 and 5). The PCR product (353 bp) aligned perfectly with the product of the E6 gene from Caski cervical carcinoma cells (lane 11), while lane 12, containing a negative sample, produced no such fragment. Note that all samples were analysed on high-resolution Visigels to precisely size fragments. Even prolonged cycling of the prostate samples (up to 45 cycles) and intensification of signal by hybridisation of the products with an E6-specific probe [Jalal et al., 1992] were unable to show presence of any HPV type 16 within the biopsies.

To check that human DNA was present within the samples, all samples were subjected to amplification with a pair of primers used consistently in our laboratory to detect the human HPRT gene (which produces a DNA amplification product of 267 bp). As shown in Figure 1B, in all cases except lane 3, a strongly positive signal was obtained. The negative control (lane 12) was

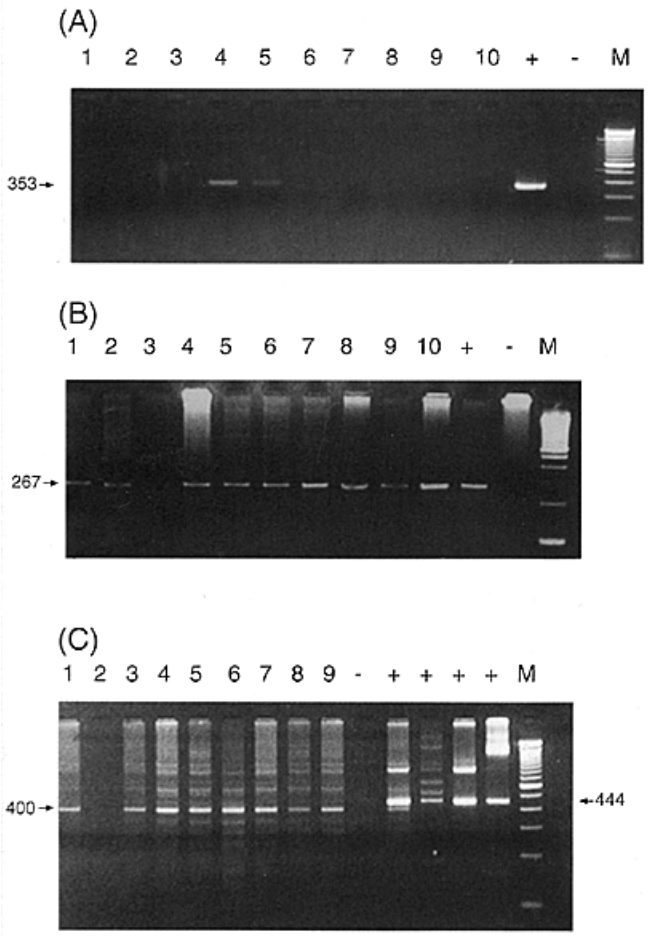

Fig. 1. Detection of HPV DNA by type-specific and general HPV primer PCRs. **A:** HPV 16-specific primers from the E6 open reading frame. **Lanes 1–3, 6–10:** Prostate biopsy DNA **lanes 4, 5:** cervical carcinoma DNA; **lane 11:** positive control Caski DNA; **lane 12:** negative control, **lane 13:** size marker DNA (100 bp ladder). **B:** HPRT cell gene-specific primers. **Lanes 1-3, 6-10:** prostate biopsy DNA; **lanes 4, 5:** cervical carcinoma DNA; **lane 11:** positive control Caski DNA; **lane 12:** negative control (plasmid DNA); **lane 13:** size marker (100 bp ladder). **C:** HPV general primers from the E1 open reading frame. **Lanes 1-9:** prostate biopsy DNA; **lane 10:** negative control; **lane 11:** Caski DNA; **lanes 12-14:** HPV 6, 11, 18 plasmid DNA (100 pg); **lane 15:** size marker DNA (100 bp ladder). All samples analysed on Visigels as recommended by the manufacturers.

again negative, and Caski DNA in lane 11 was confirmed as being of human origin by this PCR reaction. An equally sensitive primer pair for the E2 gene of the HPV type 16 was also applied to the samples and again the only positive results were seen with the cervical carcinoma controls (data not shown).

To test the hypothesis that the virus type detected within the prostate cancers may well be an atypical HPV, consensus primers from the E1 gene of high-risk HPV types were therefore employed. These primers have been used in many studies of human cervical carcinomas to detect HPV type 16 and others. As shown in Figure 1C, the majority of samples produce multiple bands when the consensus primers were used at the recommended low annealing temperature of 37°C. Under these conditions, several PCR products were produced by the prostate DNA. The negative control remained negative (lane 10) but the positive controls, including DNA from Caski (HPV16-positive cervical carcinoma) cells (lane 11), cervical carcinoma samples (not shown), and pure HPV DNAs (lanes 12–14), produced a product which was marginally higher in molecular weight than the major product present in the prostate DNAs.

To test whether the prostate-specific PCR products represented a deletion mutant of HPV present within the prostate samples and not within the cervical samples, we purified the major 400 bp PCR product and subjected it to restriction endonuclease digestion with a number of different restriction endonucleases. The major distinguishing factor between the different products, apart from their molecular weight (444 bp from cervical carcinoma and approximately 400 bp from prostate), was the presence of a HindIII site located approximately 120 bp into the PCR product. No such HindIII site exists within HPV16 [Seedorf et al., 1985].

Accumulated data for PCR reactions with all of the various primers and incorporating clinical data are shown in Table I. From this it can be seen that all samples were negative for the HPV16-specific primers whereas the consensus primers gave positive results in a large number of individual biopsies.

To confirm finally that the major products from the prostate tissues were HPV derived, the 400 bp PCR products were cloned into the vector pT7blue and subjected to direct sequencing from both ends of the insert. The sequence data from this are shown in Figure 2A where the HindIII site is marked. In addition, the location of the general purpose primers is indicated in hatched boxes at appropriate ends of the sequence. The master sequence from HPV16 prototype (Seedorf et al., 1985] is shown in Figure 2B for comparison, together with translation products in the correct sense for the E1 open reading frame.

DISCUSSION

PCR analysis of biopsies of human prostate cancer taken by transurethral resection, snap frozen, and sectioned under sterile conditions taking maximal precautions to minimise the possibility of cross-contamination between samples gave excellent DNA for PCR but proved negative when assayed for the presence of HPV type 16 DNA. This is entirely in agreement with a number of recent studies [Effert et al., 1992; Serfling et al., 1992], but disagrees fundamentally with the earlier published studies [Ibrahim et al., 1992; Anwar et al., 1992b; McNicol and Dodd, 1991; Rotola et al., 1992] as reviewed by Cuzick [1995]. To explore whether a variant HPV is responsible for the positive result, we subjected the same DNAs to analysis with the general PCR primers originally designed by van den Brule et al. [1990] at low annealing temperatures (but not at high annealing temperatures). This gave a product of comparable size to that given by both cervical carcinoma controls and the Caski cell line when analysed by agarose gel electrophoresis. The other multiple products detected after

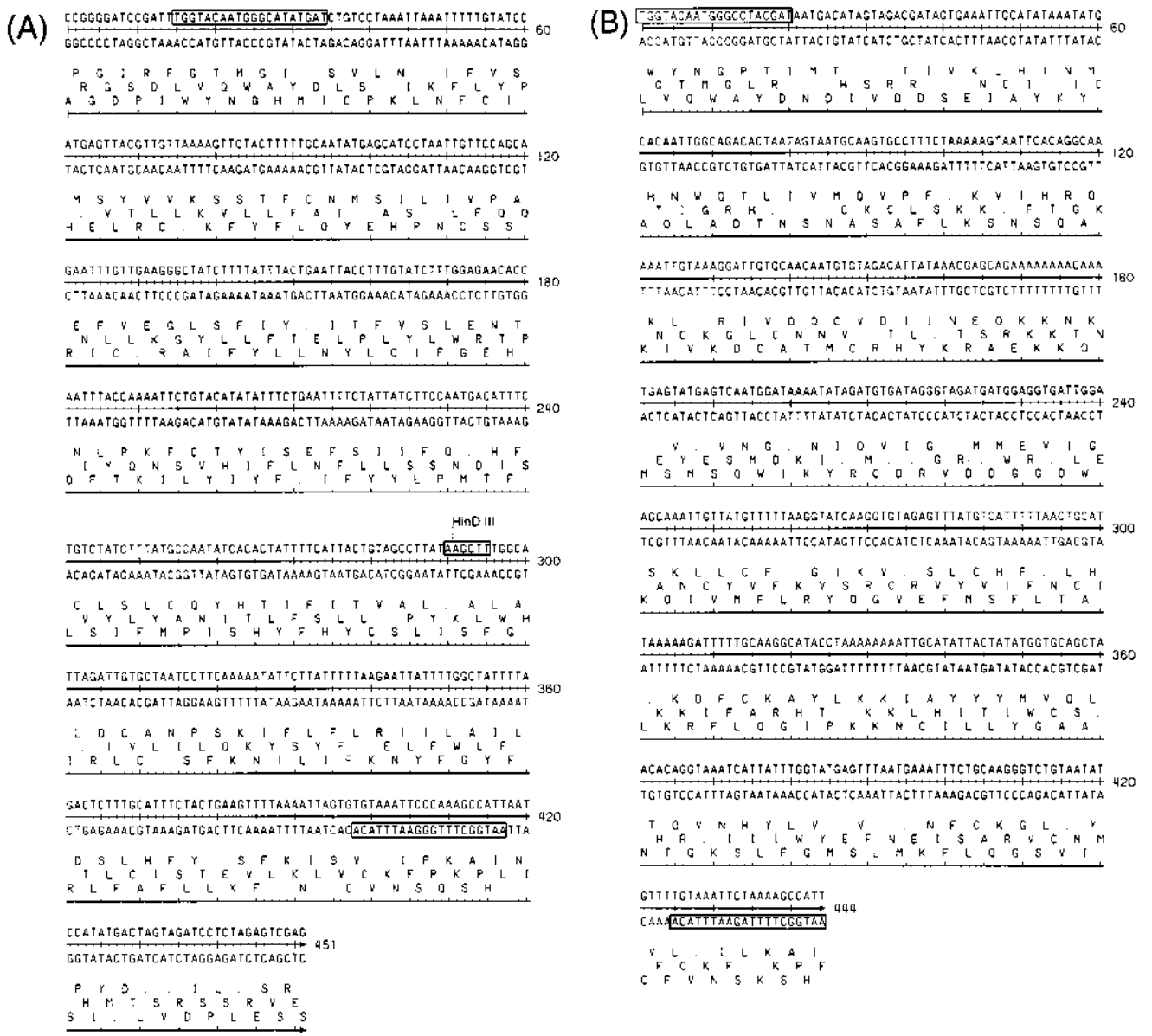

Fig. 2. Sequence comparisons between prototype HPV16 and sequenced 400 bp PCR product from prostate biopsies: **A:** Fragment of HPV16 E1 open reading frame amplified by the general primers. **B:** Sequence of 400 bp PCR product and predicted amino acid sequence in all reading frames. Open reading frames are shown in single letter codes below the double-stranded DNA sequence. Stop codons are indicated by dots in all reading frames. PCR primers are boxed at the 5′ and 3′ ends of the sequences.

the low temperature annealing were due to mismatch priming, as they were absent from purified HPV DNA and HPV-positive cervical carcinoma DNA products produced at high annealing temperatures. However, when analysed on high-resolution Visigels, the product appeared to be some 40 bp shorter than that of the prototype. It also contained a HindIII site not present in the prototype DNA (see Fig. 2). On sequencing of this cloned product it was shown that the two PCR primers were employed in their correct orientation and flanked the sequence cloned. The location of the HindIII site in the product was also confirmed. However, on examination of the sequence and translation in all three reading frames in both orientations it could be shown that this product was incapable of coding for any protein at all, let alone the E1 protein as is normally the case with the product of the GP1 and GP2.

What is the origin of this aberrant PCR product? Firstly, the complexity of the patterns as shown in Figure 1C indicated the possibility of mispriming, or a RAPD type pattern, primed at both ends by the same primer [Williams et al., 1990]. The sequence analysis indicated that this was not the case. A more likely explanation is the presence of RNA contamination in the prostate DNA as a consequence of the preparation method used, which has brought the two primer se-

quences into close apposition. This explains the identical patterns observed in the prostate, compared to the various cervical carcinomas, where gene expression patterns will differ and where DNA samples were considerably more pure. Thus, we would conclude that HPV16 is not present within human prostatic carcinomas and that any products found by the study of general primers under low temperature annealing conditions must be viewed as spurious and not of HPV origin.

Since the study was carried out in a double-blind manner with randomly inserted positive controls, we can be certain that we have minimised the chances of cross-contamination between samples and that our negative result is indeed truly negative. We suggest that HPV16 does not have a major role to play in adenocarcinoma of the prostate and that the prostate itself may not be a suitable reservoir as hypothesised in some studies for HPV16. This does not exclude the possibility of the presence of other HPV types which fail to react with the GP pair.

Our results would therefore agree with those of Sinclair et al. [1993]. As summarised recently by Cuzick [1995], we suggest that a high positive rate of HPV DNA and in fact RNA detection might well have occurred due to contamination of biopsy samples during the transurethral biopsy procedure. This would involve crossing an area very likely to contain HPV16 based on studies by [Della Torre et al., 1992], who found significant levels of HPV in male genital tissues.

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